Developmental Stability of the Tonotopic Organization of the Chick's Basilar Papilla

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In the past, the damage patterns produced on the papilla of the chick by loud pure tones of different frequencies have been used to study the development of sound analysis by the hair cells of the basilar papilla. One conclusion from these data was that the best response frequency of individual hair cells changes substantially with age. However, this method has been criticized as unreliable. Now experiments have been performed in which single characterized nerve fibers were stained with horseradish peroxidase to permit the unequivocal localization of specific frequency responses in the papillae of chicks of different ages (2 and 21 days after hatching). There was no statistically significant change in the tonotopic organization of the papilla between the two groups of animals.

ON THE BASIS OF ACOUSTIC TRAUma and brain mapping experiments (*1*), it has been suggested that the developmental changes in damage patterns on the basilar papilla of chicks indicate that the hair cells change their preferred response frequencies with age. This conclusion implies that the entire frequency distribution on the basilar papilla changes with time (*2*). Because noise-damage experiments are difficult to interpret unambiguously, we have carried out a series of experiments in which we marked frequency-characterized primary auditory neurons with horseradish peroxidase (HRP) in newly hatched [postnatal day 2 (P2)] and older [postnatal day 21 (P21)] chicks to trace directly the peripheral origins of cells of the same characteristic frequency. The frequency distribution on the basilar papilla for birds of these two age groups was not statistically different.

Our experiments were carried out on 29 Selected Leghorn chicks (*Gallus gallus domesticus*) of age P2 (±1 day) and 24 chicks of the identical breed of age P21 (±1). They were anesthetized (*3*), warmed, and respirated via a tracheal cannula with a continuous stream of moist air. After immobilizing the head, we exposed the cochlear ganglion by the same technique as that reported for the starling (*4*).

We recorded in different regions of the ganglion with glass microelectrodes (*5*). Directly after primary auditory units were encountered, their frequency response was investigated (*6*). After electrophysiological characterization, we attempted to fill the nerve fiber with HRP (*7*). After subsequent survival of the animal for at least 2 hours, the chick was perfused, and the cochlea was removed and processed to allow visualization of the labeled fibers (*8*). The cochleae were embedded in Spurr, and, after they were measured and scale drawings of the stain and papilla in the whole preparation were made, they were cut into 20-μm sections. We attempted to cut all cochleae with the same orientation to minimize possible errors from different section orientations. We took great care to accurately localize the marked fibers (*9*).

Whereas, in a few cases marked cells could be easily identified in whole mounts, the dark staining of endogenous enzymes in hair cells usually made this impossible. Thus, the positions of stained nerve fibers in the papillae were, in general, only determined from careful serial reconstructions of sectioned material. If we add the values of all potential sources of error in our technique, we arrive at a maximal error of about 10% of the papillar length. However, we estimate the typical case to be less than half of this.

In P2 animals, we found 13 cases of unambiguously stained (well-localized) fibers or small groups of fibers. In two cases, the stain was also found in the innervated hair cell. HRP was taken up differently in P21 animals (*7*), and we found only one case of single-fiber staining. In P21 animals, we measured eight marked regions, each of which did not exceed 10% of the length of the papilla (the actual extents are given in the figure as bars). Cases of more extensive staining were eliminated from the analysis, even though their distribution was consistent with the other data.

We could generally follow fibers from their synaptic contact with a hair cell to the habenula perforata. When one or only a few fibers were stained, the fibers contacted only a single tall hair cell (*10*). This is interesting because of the parallels that can be drawn between tall and short hair cells of birds and inner and outer hair cells of mammals, respectively (*11*).

For comparison, we present the data on the localization of stained fibers under hair cells as a percentage of the length of the papilla from the apical end (Fig. 1, A and B). In each case, we calculated both simple regression lines and the polynomial best fit to the data (*12*). The lines in the figure represent the best-fit polynomial (second order) and are compared in Fig. 1C. The greatest difference between the curves is at low frequencies. At the 500-Hz position, the difference represents 5.8% of the length of the papilla. These values are well within the potential measurement errors, and the two curves are not significantly different (*12*). In Fig. 1C, the data of Rubel and Ryals (*1*) are also reproduced. We did not see a difference comparable to that seen by Rubel and Ryals—more than 20% of the papillar length for the ages E20 (just before hatching) to P30 or 10% between P10 and P30.

Simple regression slopes of these data represent a frequency distribution of about

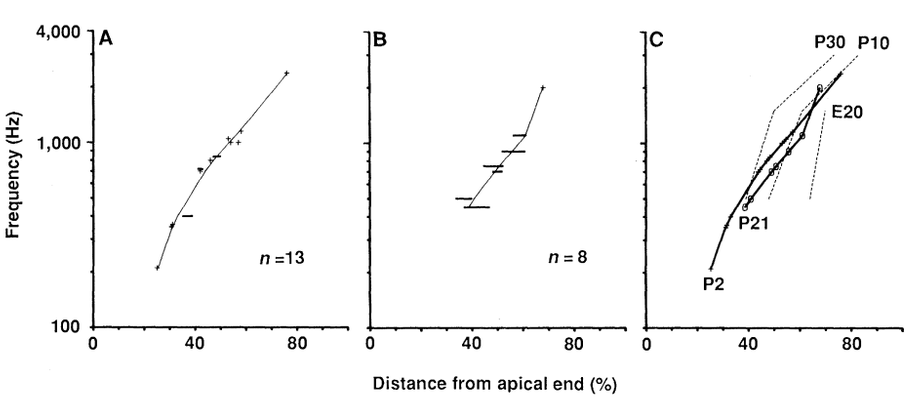

Fig. 1. Distribution of characterized, HRP-stained auditory nerve fibers in the papillae of (**A**) P2 and (**B**) P21 animals expressed in terms of their position with respect to the apical end. Crosses indicate that staining was present in only a single section. Bars indicate the extent of marked fiber regions in cases where cells in more than a single section were stained. In (B) there are two overlapping data points at 900 Hz. Second-order polynomial fits are plotted for each set of data (*12*). In (**C**) these two functions (solid lines) are compared to the data of Rubel and Ryals (dashed lines labeled E20, P10, and P30).

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0.5 mm per octave, although the P2 data do suggest a break in the slope near 800 Hz. This function does not differ greatly from that determined for the chicken by von Békésy, although his data indicate a somewhat steeper slope (*13*).

We conclude from our data that the distribution of the best frequencies of tall hair cells in the chick basilar papilla does not change significantly between the ages P2 and P21. We do not have any data from fibers innervating short hair cells, so we cannot discuss possible developmental changes in that hair cell area. The fact that we do not know the functional significance of the presence of two hair cell populations in birds complicates the comparison of our data with those of the noise-damage experiments.

At low levels of damaging sounds, Cotanche *et al.* (*2*) describe damage to the papilla only in an area of hair cells abneural to the inner edge of the basilar membrane. The nerve fibers described in our study innervated hair cells neural to this area, so that we may in fact be describing the development of a different group of cells to those primarily damaged by loud sounds, which presumably contributed to the total hair cell losses described by Rubel and Ryals (*1*). According to Cotanche *et al.* (*2*), the changes that can be observed in sound-induced damage patterns in the chick such as those also observed by Rubel and Ryals are correlated more with stimulus intensity and a change in the middle-ear admittance than with age. This change in middle-ear admittance is such that the effective intensity of a standard stimulus changes with age.

Whether there is a change in the tonotopic organization of the papilla before hatching cannot be determined from our results. Our data do indicate, however, that the source of the well-known increase in sensitivity to higher characteristic frequencies in the developing chick after hatching (*14*) must be sought in phenomena that do not require a change in the peripheral distribution of characteristic frequencies.

REFERENCES AND NOTES

1. E. E. Rubel and B. M. Ryals, *Science* **219**, 512 (1983); W. Lippe and E. W. Rubel, *ibid.*, p. 514; *J. Comp. Neurol.* **237**, 273 (1985). Also, age-related shifts in the best frequency of microphonics of the midbasal region of the gerbil cochlea have been attributed to an age-dependent shift in micromechanical response [D. M. Harris and P. Dallos, *Science* **225**, 741 (1984)].
2. That the spatial coding of frequencies in the vertebrate basilar papilla may change with time might not be unexpected in view of the systematic changes with age induced in similarly tuned and homologous fish electroreceptors [H. H. Zakon and J. H. Meyer, *J. Comp. Physiol.* **153**, 477 (1983)]. D. A. Cotanche, J. C. Saunders, and L. G. Tilney [*Hear. Res.* **25**, 267 (1987)] have, however, recently repeated the noise-damage experiments and came to a quite different conclusion. In addition, a further study also claimed there is a shift in damage patterns, but of only 8% of the length of the basilar papilla, and this only within a 2-day period at hatching [H. Cousillas and G. Rebillard, *Hear. Res.* **19**, 217 (1985)].
3. P2 birds received initial doses of Nembutal (8 mg per kilogram of body weight) and cloral hydrate (113 mg/kg); P21 animals received 14 and 210 mg/kg, respectively. Body temperature was held at 39 ± 2°C.
4. G. A. Manley, O. Gleich, H.-J. Leppelsack, H. Oeckinghaus, *J. Comp. Physiol.* **157**, 161 (1985). We observed the strong tonotopic organization previously seen in the starling cochlear ganglion. As in the starling, it is physically difficult to reach the high-frequency end of the ganglion. Also, in older chicks, the low-frequency end of the ganglion is obscured by a thin bony plate.
5. Thin-walled glass microelectrodes were filled at the tip with a 10% solution of HRP in tris buffer. Cells were successfully labeled when the electrode resistance lay between 30 and 200 Mohms.
6. For this purpose, we determined threshold-response curves for the individual fibers using either audiovisual criteria or with a fully automatic procedure that counted responses at many points within a large matrix of frequency and sound pressure. Apart from their responses to a white-noise search stimulus, cells were detected by means of dc shifts in the recording trace. These shifts were not large (−10 to −25 mV), and the action potentials were 2 to 6 mV in size, so that the recordings are considered "quasi-intracellular."
7. We used positive current pulses (50 msec, 10 pulses per second, for a total current of 25 to 125 nA-minutes). The uptake of HRP in the older animals was more diffuse than in the younger animals and seldom produced as intensely stained fibers as in P2 animals.
8. The birds were perfused through the heart with warm bird Ringer solution, then cooled glutaraldehyde (2.5% in phosphate buffer at *p*H 7.4). The entire lagena was then removed and placed in the fixative for 30 minutes. After repeated washings in the buffer, the lagenae were stored for 1 to 3 days in cooled buffer before further treatment. The limbus, basilar papilla, and nerve were carefully freed from bone and the tegmentum vasculosum and macula lagenae removed. The reaction with diaminobenzidine was carried out after incubation according to the technique described by Mesulam [M. M. Mesulam, *Tracing Neural Connections with Horseradish Peroxidase* (Wiley, New York, 1982)], modified for whole mounts.
9. The bird basilar papilla is not straight, but is curved and twisted. To minimize errors, each cochlea was drawn and measured before and after embedding, with the calibrated eyepiece of a microscope. We took an average of two measurements of the dimensions and the curvature, starting from the basal end, and two from the apical end, together with reconstructions of the cochlea from serial sections. The strongest curvatures are present in the high-frequency (basal) region, where we did not stain any cells, so we measured fiber locations with respect to the apical end. As the fixed P2 papillae were very similar in length (3.5 ± 0.12 mm), we treated them all as equally long. This was not possible in the P21 animals, where the variation in length was greater (3.71 ± 0.35 mm).
10. Only in cases where large areas of the papilla were stained (cases not used in this analysis) were marked fibers found that branched and innervated a number of short hair cells. In those cases where short hair cell fibers were stained, we also found stained tall hair cell fibers. There were no cases in which only short hair cell fibers were stained. If we ignore cases of overstaining, we thus apparently recorded exclusively from tall hair cell fibers.
11. See G. A. Manley [*Auditory Frequency Selectivity*, B. C. J. Moore and R. D. Patterson, Eds. (Plenum, New York, 1986), p. 63] for a discussion. Although there are few paleontological or experimental data supporting the idea, it has often been assumed from the relative positions of the tall and short hair cells on the papilla basilaris of birds that they are functionally equivalent to the inner and outer hair cells of mammals, respectively. In mammals, stained afferent auditory nerve fibers have been found only to innervate the inner hair cells [M. C. Liberman, *Hear. Res.* **3**, 45 (1980)]. Our data thus support the supposition of functional equivalence between the two groups of hair cells in these vertebrates.
12. Polynomial fits were carried out with the BMDP statistics package [W. J. Dixon, Ed., *BMDP Statistical Software* (University of California Press, Berkeley, 1985)]. Whereas for the P2 animal data the second-order fit was obviously the best, there was little improvement in the case of the P21 animal data. We have used the second-order fit in Fig. 1, A and B, for comparison purposes. For P2, R^2 was 0.98; for P21, 0.924. Using simple linear regression equations, we established that the two functions are not significantly different (*t* test for comparing simple regression equations, $t = 0.53$) [J. H. Zar, *Biostatistical Analysis* (Prentice-Hall, Englewood Cliffs, NJ, 1974)]. When more than one fiber was stained, we used the center of the stained area as a point in the calculations.
13. G. von Békésy, *Experiments in Hearing*, E. G. Wever, Transl. (McGraw-Hill, Chicago, 1960).
14. L. Gray and E. W. Rubel, *J. Acoust. Soc. Am.* **77**, 1162 (1985); G. Rebillard and E. W. Rubel, *Brain Res.* **229**, 15 (1981); J. C. Saunders, R. B. Coles, G. R. Gates, *ibid.* **63**, 59 (1973).

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